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Quantitative Differences in the Distribution of Zidovudine Resistance Mutations in Multiple Post-Mortem Tissues From AIDS Patients

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Replication of HIV introduces errors into the genome which are responsible for conferring a growth advantage over wildtype virus when drugs such as zidovudine (ZDV) exert a selective pressure. The molecular basis for HIV-1 resistance to ZDV has been mapped to codons 41, 67, 70, 215 and 219 of the reverse transcriptase gene both in vitro and in clinical samples of blood. This study has investigated the relationship between the quantitative prevalence of ZDV resistance in multiple organs of the same individual. Proviral HIV-1 load was measured by quantitative-competitive PCR in 90 samples from organs of 11 patients dying with AIDS. Nine of these patients had been prescribed zidovudine. The distribution of wildtype and mutant sequences at the positions 41, 67, 70, 215 and 219 of the reverse transcriptase was assessed using a point mutation assay. The results showed that the highest proviral loads were predominately found in lymph node, spleen and lung and there was a significant association between viral load and resistance to ZDV (P = 0.008). Inter-organ distribution of wildtype and mutant sequences at codons 41, 67, 70, 215 and 219 was frequently not uniform and in some patients differed markedly between the lymphoreticular system and other organs. These results demonstrate that treatment of HIV-1 infection with zidovudine does not exert uniform selective pressures in multiple organs. These findings have implications for the interpretation of resistance data and design of treatment strategies for HIV, arguing in particular that alterations in therapeutic regimens should consider the likelihood of different resistance patterns being present in multiple sites within the same individual. ***J. Med. Virol. 55:138–146, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: HIV-1; polymerase chain reaction; mutation; resistance

INTRODUCTION

The pathological processes that result in patients with human immunodeficiency type 1 infection (HIV-1) developing AIDS are still poorly understood but this has not prevented the development and clinical use of anti-retroviral therapeutic agents. Until recently, the most widely used inhibitor of HIV-1 has been zidovudine (ZDV) (3′-azido-3′- deoxythymidine) which is a potent inhibitor of the HIV-1 reverse transcriptase in vitro and results in a mean 0.5–1.0 $\log_{10}$ reduction of plasma HIV load in patients [Piatak et al., 1993; Loveday et al., 1995]. Clinical trials have shown that ZDV is effective in decreasing the incidence of opportunistic infections and reducing mortality in patients with advanced disease [Fischl et al., 1987; Hamilton et al., 1992; Kinloch-de Loes et al., 1995] although it appears to offer limited benefit in patients with higher CD4 counts when used as monotherapy [Volberding et al., 1990; Cooper et al., 1993; Concorde Coordinating Committee 1994]. It has also been demonstrated that treatment with ZDV can reduce by two-thirds the rate of transmission from mother to baby [Connor et al., 1994].

The appreciation of the dynamics of HIV-1 infection in the human host provides the basis for understanding the rapid emergence of resistance to anti-retroviral drugs [Embretson et al., 1993; Pantaleo et al., 1993; Ho et al., 1995; Wei et al., 1995]. Ex vivo studies and site directed mutagenic studies have shown that ZDV resistance segregates with mutations at codons 41, 67,

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TABLE I. Demographic and Clinical Characteristics of the Patients Studied

Patient (sex, age at death)	Treatment regimen and duration	CD4[a]	CD4 (at death)	Ols/Malignancies
1 (F 38y)	ZDV 250 mg BD 14 months	60	0	PCP X3, MAI, NHL
2 (M 63y)	ZDV 200 mg TDS 29 months	250	10	MTB,HCMV
3 (M 53y)	ZDV 400 mg TDS 36 months	200	10	Toxoplasma, NHL
4 (F 38y)	ZDV 200 mg TDS 47 months	180	0	HCMV
5 (M 46y)	ZDV 250 mg BD 36 months	120	0	PCP, wasting
6 (M 42y)	ZDV 250 mg BD 15 months	50	10	HCMV, KS
7 (M 38y)	ZDV 250 mg QDS 33 months	205	0	HCMV, KS, PCP
8 (M 55y)	ZDV 200 mg TDS 32 months	130	10	Wasting, Cryptosporidiosis, Cerebral atrophy
9 (M 32y)	ZDV 100 mg QDS 8 months	50	20	ITP. Haemorrhagic Cystitis
10 (M 36y)	NONE	N/A	10	N Meningitidis
11 (M 37y)	NONE	N/A	10	MAI, HCMV

ZDV = Zidovudine; PCP = Pneumocystis carinii pneumonia; MAI = Mycobacterium avium intracellularae; NHL = Non-Hodgkin's lymphoma; MTB = Mycobacterium tuberculosis; HCMV = Cytomegalovirus; KS = Kaposi's sarcoma; ITP = Idiopathic thrombocytopenic purpura.
[a]At the start of ZDV therapy (cells/μl).

70, 215 and 219 of the HIV-1 reverse transcriptase (RT) gene [Larder et al. 1989; Kellam et al., 1992]. The pathway towards high level resistance is complex and results in a series of linked mutations which result in synergistic modulations in the level of phenotypic resistance to ZDV [Larder et al., 1991; Richman et al., 1991; Boucher et al. 1992]. To date, the majority of studies aimed at identifying in vivo resistance of HIV-1 to ZDV and to other anti-retroviral agents have employed phenotypic and genotypic analysis of HIV-1 strains present in plasma or populations of peripheral blood mononuclear cells (PBMC). The relationship of strains circulating in the periphery to strains present in organs is complex and undefined. For example, studies have demonstrated the presence of phylogenetically and phenotypically distinct variants at a number of HIV-1 genetic loci in different organs including blood and within the same organ or organ system [Dellasus et al., 1992; Kaye et al., 1992; Smith et al., 1993; Ball et al., 1994; Ait-Khaled et al., 1995; Wong et al., 1997]. In the light of these data we have investigated the distribution of HIV-1 RT resistance mutations in patients prescribed ZDV for varying lengths of time and have correlated resistance with viral load in each organ since active replication is a pre-requisite for the generation of diversity. We reasoned that such information would help understand the relationship between ZDV resistance in the lymphoreticular system and resistance present in other organs and could have implications for the continued use of ZDV and other antiretroviral agents either in combination or as single therapeutic agents for the treatment of HIV-1 infection.

METHODS

Patients and Samples

Tissues from organs and cardiac blood were taken at post-mortem examination from 11 HIV-1 infected individuals who died with AIDS defining illnesses (CDC Class IV). Post-mortem examinations took place within 5 days of death and tissue samples were stored at −70°C. Pre-mortem blood samples were available for analysis from 4 patients. DNA was extracted from tissues by dissecting finely 5 mm × 5 mm × 5mm pieces followed by 3 washings with PBS (1 ml) to remove residual blood. Macerated samples were subsequently washed 3 times for 2 min in 10 mM Tris-HCl (pH8) and 0.5% SDS and then incubated overnight at 37°C in 1 ml of extraction buffer (10 mM Tris-HCl pH8, 10 mM EDTA, 1% SDS and 50 ng/ml proteinase K). DNA was extracted twice with an equal volume of buffer saturated phenol and a further 2 times with buffer saturated phenol-chloroform. The DNA was precipitated with ethanol and the dried DNA pellet was dissolved in 150 μl of sterile distilled water. DNA from pre-mortem and cardiac blood was extracted using commercially available blood DNA extraction kits (Qiagen, Digene Laboratories, Germany) according to the manufacturer's instructions.

Quantitative PCR for HIV-1 Proviral DNA

One μg of DNA extract was subjected to nested PCR with primers to the gag region of HIV-1. The primers possessed the following sequences: P1, 5′GAGGAGCCACCCCACAATATT; P2, 5′TAGGTGGATTATTTGTCATCCA and hybridised to positions 1317–1337 and 1553–1554 of the HXB 2 prototype sequence. The nested primer pair were as follows: I1, 5′TGCTAAA-

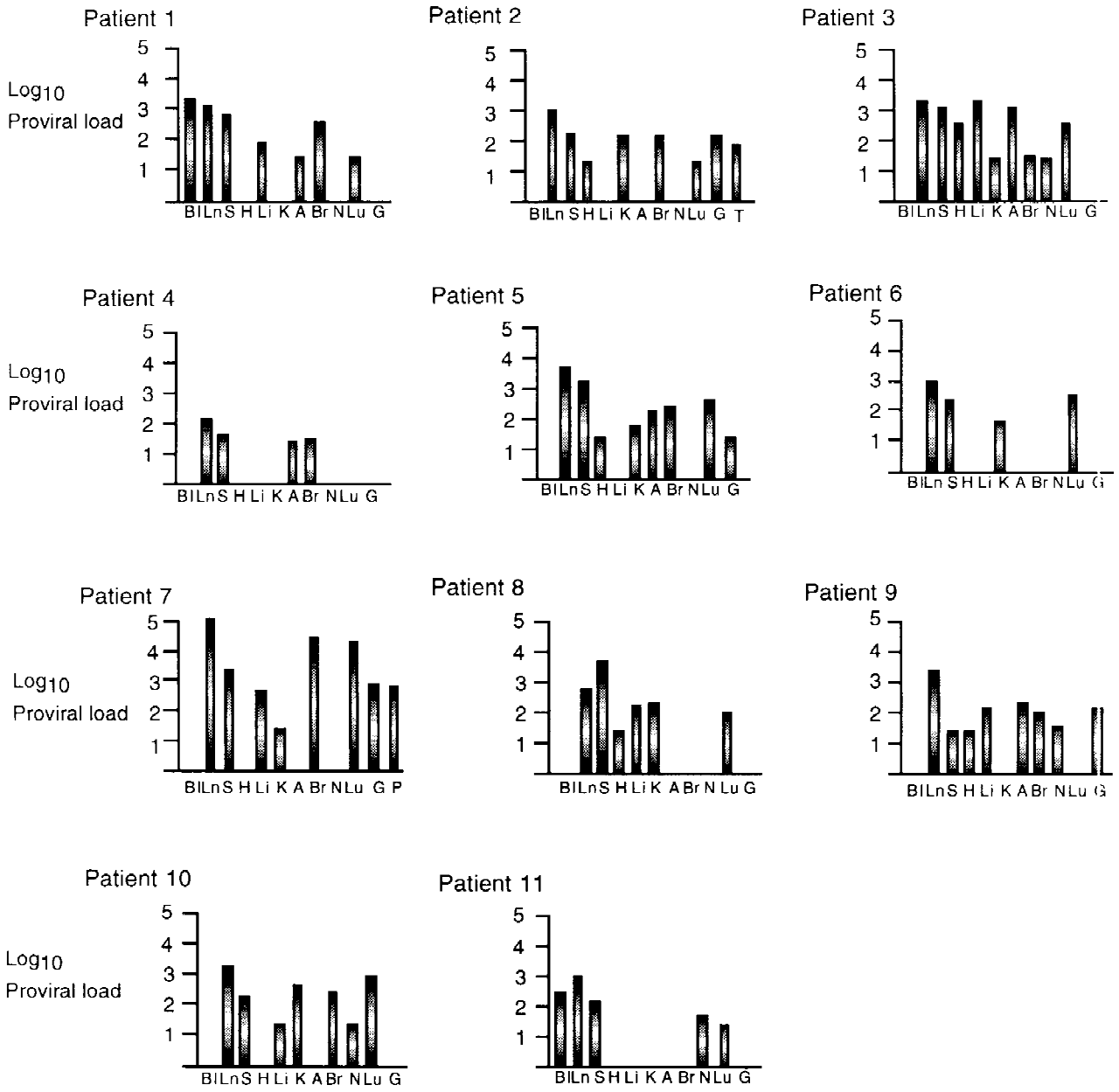


Fig. 1. Distribution of proviral HIV loads in multiple organs of 11 patients dying with AIDS. Viral load is shown as $\log_{10}$ values. Abbreviations: Bl, blood; Ln, lymph node; S, spleen; H, heart; Li, liver; K, kidney; A, adrenal; Br, brain; N, nervous tissue (spinal cord and ganglion samples); Lu, lung; G, gastrointesinal tract (including colon and duodenum). Gastrointestinal tract was unavailable for patients 1 and 5 while cardiac blood was only obtained from patients 1 and 11.

CACAGTGGGGGGA; I2, 5′CCTGAAGGGTACTAGTAGTT and hybridised to positions 1346–1365 and 1521–1540 of the HXB2 prototype sequence. Each PCR amplification contained a known quantity of a control plasmid which was identical to the P1/P2 amplicon except for the mutation of two nucleotides to generate a *Sma*1 restriction site. Either 10^4, 10^3 , 5×10^2 or 10^2 copies of the control sequence were added to one of four PCR reactions for each tissue sample. The 1st round of PCR proceeded for 35 cycles whilst the 2nd round was truncated to 15 cycles. Under these conditions the PCR reaction remains exponential. PCR reaction products were digested with *Sma*1 according to established procedures and products resolved by electrophoresis through an 8% polyacrylamide gel. DNA in the gels was then visualized using a silver staining kit (Promega) according to the manufacturer's instructions. A positive image of the stained gel was obtained using EDF film (Kodak) and the relative intensities of the target amplicon and control amplicon determined by scanning densitometry. HIV-1 proviral loads were expressed as genome equivalents per μg total DNA.

Point Mutation Assay

The point mutation assay for the analysis of the distribution of wild type and mutant nucleotides at codons

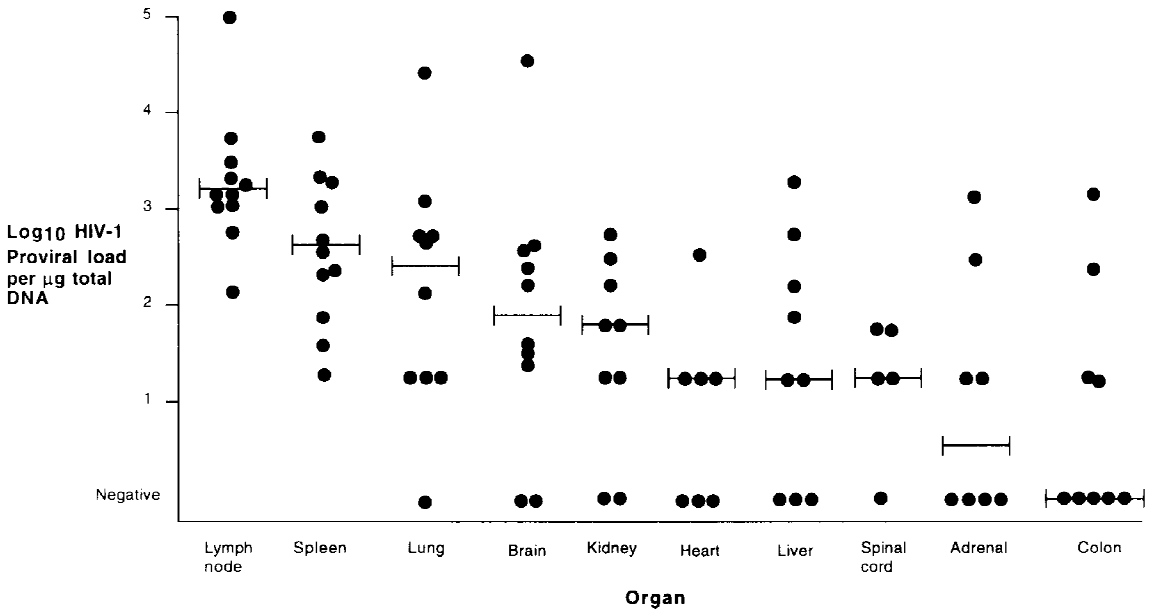


Fig. 2. Interorgan distribution of HIV-1 proviral load in multiple organs obtained from 11 patients dying with AIDS. The median proviral load for each organ is shown. The proviral loads in lymph node and spleen were significantly higher than all other organs ($P < 0.01$) except in the case of spleen and lung where there was no significant difference.

41, 67, 70, 215 and 219 of the reverse transcriptase gene was carried out as described previously [Kaye et al., 1992]. Briefly, 808 base pair biotinylated PCR products amplified by a nested PCR from the 5′ end of the pol gene were immobilised using streptavidin coated microtitre plates and annealed to oligonucleotide probes hybridizing adjacent to each residue of interest. Extension of the primer in the presence of ^{35}S labelled dATP, dCTP, dGTP or dTTP using the Klenow fragment of DNA polymerase I followed by liquid scintillation counting of the extended products allowed the distribution of nucleotides at the position of interest to be calculated. Data are shown as the percentage of total radioactivity distributed between mutant or wildtype nucleotides. Differences of >10% between wt and mutant were designated significant on the basis of previous experience with these types of assay [Kaye et al., 1992; Bowen et al., 1997] whilst values of wildtype or mutant alleles which were less than 10% were not deemed to be significant.

Statistical Analysis

The data were analysed for significance by the Mann–Whitney *U*- test and Spearman's rank correlation coefficient as appropriate.

RESULTS

Ninety individual organs from 11 patients dying with AIDS were examined for the presence of HIV-1 resistance mutations at codons 41, 67, 70, 215, 219 by a point mutation assay and for HIV-1 proviral DNA using a quantitative-competitive PCR method. The details of the patients are summarized in Table I. Nine patients had been treated with ZDV for between 8 and 47 months (mean duration 27.25 ± SD of 13.5 months). The mean age at death was 42.3 years. Two patients had never received ZDV and were assessed for both resistance mutations and viral load in a similar fashion.

The distribution of viral load between organs in all 11 individuals is shown in Fig. 1. Proviral DNA could be detected in the majority of tissues examined; however the predominant HIV-1 proviral reservoirs in individual patients were lymph node, spleen and lung. In patients 1 and 7 and to a lesser extent patients 9 and 10, the brain also contained relatively high proviral loads. The mean HIV-1 proviral load in all tissues examined was 260 ± 7.6 genomes/μg DNA. Assessment of proviral loads on an organ basis (Fig. 2) confirmed the patient based results shown in Fig. 1. Proviral burdens in lymph node and spleen were significantly higher than all other tissues ($P < 0.05$ and $P < 0.01$) with the exception of lung in which only the lymph node burden was significantly higher (Fig. 2).

The results of the distribution of mutant/wildtype alleles at each amino acid position associated with ZDV resistance are shown in Fig. 3. In the two patients who had not been exposed to ZDV (patients 10 and 11) there was no evidence of resistance at positions 41, 67, 70 or 219. Patient 9 who was prescribed ZDV for 8 months prior to death but, due to severe anaemia, was only able to take medication intermittently possessed a ZDV resistance profile comparable to the ZDV naive patients. However, a small but reproducible quantity of resistance genotype at position 215 (ca. 7%) was noted in this patient (data not shown). The remaining 8 patients possessed a resistance profile involving mutations at all codons although mutations at codons 41 and 215 predominated. Careful inspection of the results for the individual patients shown in Fig. 3 indicated that the distribution of mutations at each codon was not

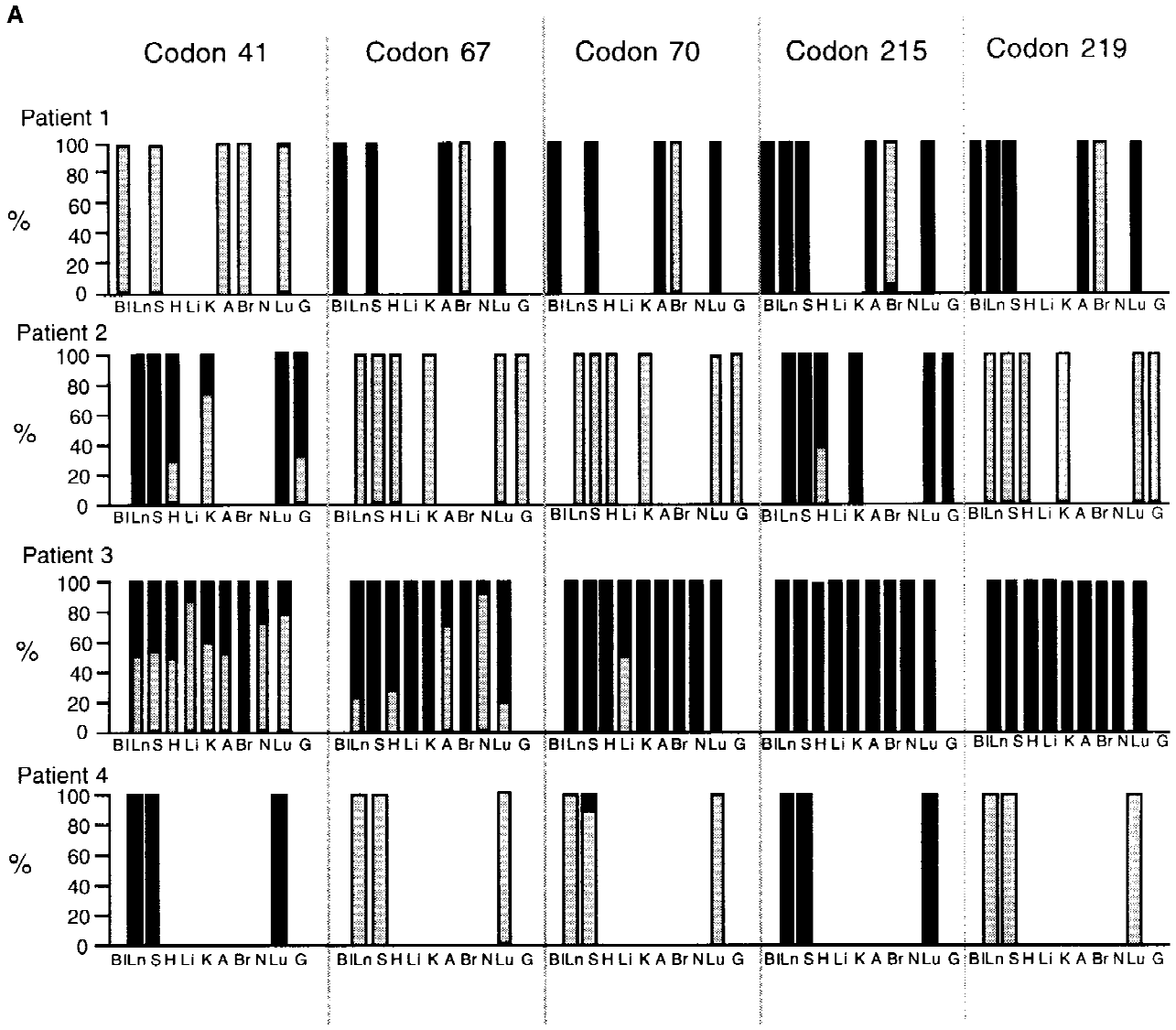


Fig. 3. Distribution of wildtype (grey bars) and mutant (black bars) sequences at codons 41, 67, 70, 215 and 219 of the RT gene in multiple organs obtained from 11 patients dying with AIDS. The percentage of wildtype or mutant sequence is indicated for each codon within each tissue analysed. Organ abbreviations are the same as those in Fig. 1. In the organs from some patients (e.g. patient 7) the point mutation assay yielded no signal for wildtype or mutant at codons 41, 67 and 70 due to a high number of mutations within the probe binding site (see Discussion). The patient descriptors correlate with those recorded in Table I.

uniform in all organs. In all patients exposed to ZDV subtle or major differences between the distribution of mutant and wild type alleles at RT amino acid positions segregating with ZDV resistance were observed. This differential distribution was most apparent in patients 1, 3, 5 and 7. In patient 1 the resistance profile in cardiac blood, lymph node, spleen, adrenal and lung were similar showing high levels of genotypic resistance at codons 67, 70, 215 and 219. In contrast, the brain sample possessed 100% wild type sequences at codons 67, 70 and 219 and 90% wild type sequence at codon 215. This patient had a proviral burden in brain of 1000 genomes/μg DNA and had been prescribed ZDV for 14 months prior to death. Similarly, in patient 7, brain and liver contained wildtype sequences at codon 219 whereas cardiac blood, lymph node, spleen and kidney contained 80% mutant at codon 219 and ganglion and lung contained 100% mutant at this position. Interestingly, both patients 1 and 7 possessed high level genotypic resistance at all positions in their peripheral blood more than 9 months before death.

In order to investigate the relationship between proviral load and presence of HIV-1 resistance to ZDV we correlated the mean % of resistance codons (the sum of the fraction on mutant at each codon analysed divided by the number of codons included in the analysis) for each organ with HIV proviral load. These data are presented in Fig. 4. Despite the high degree of scatter within the analysis, there was a statistically significant association between increasing proviral load and the presence of increasing resistance. However, it should be noted that high level resistance could still

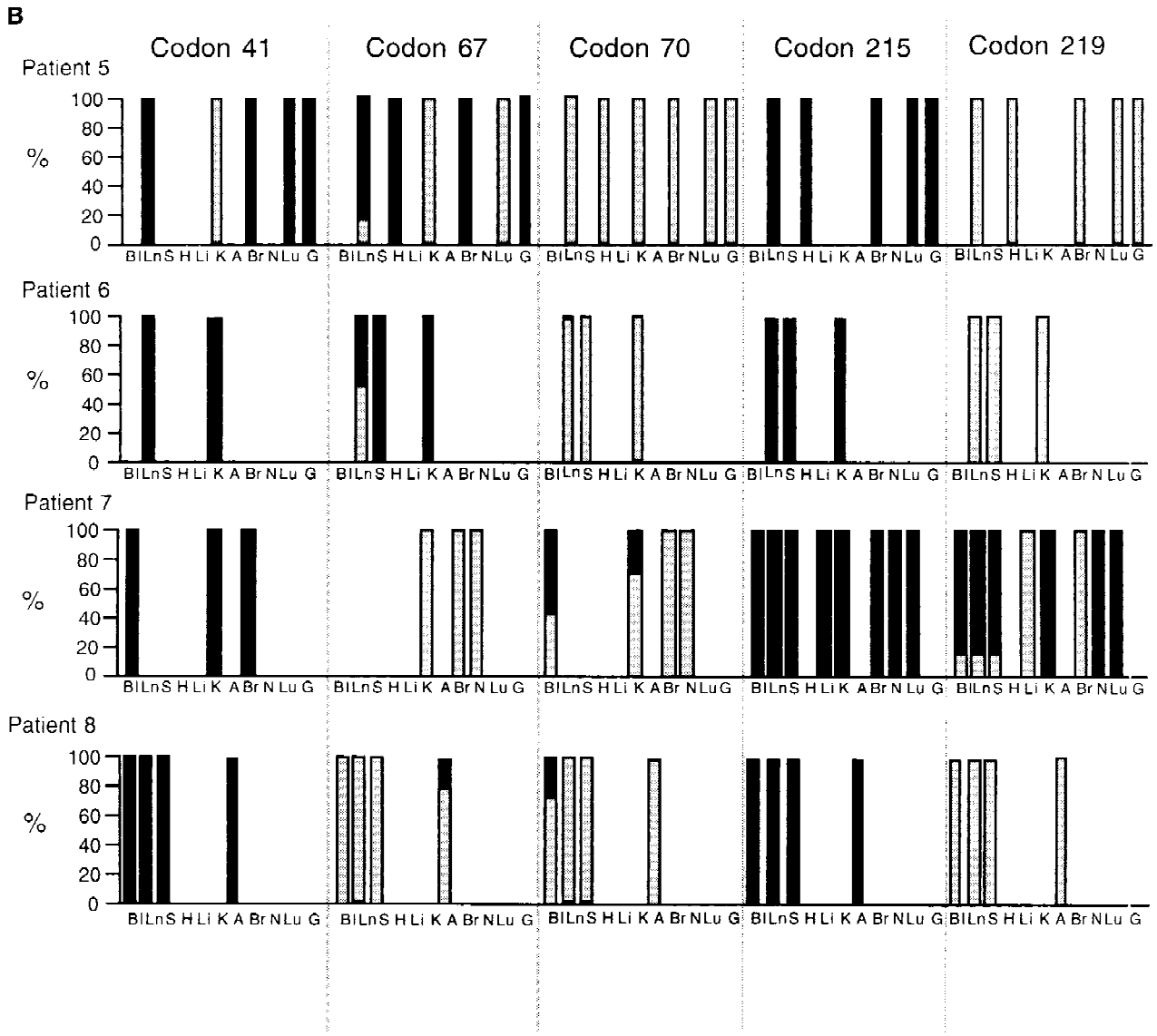
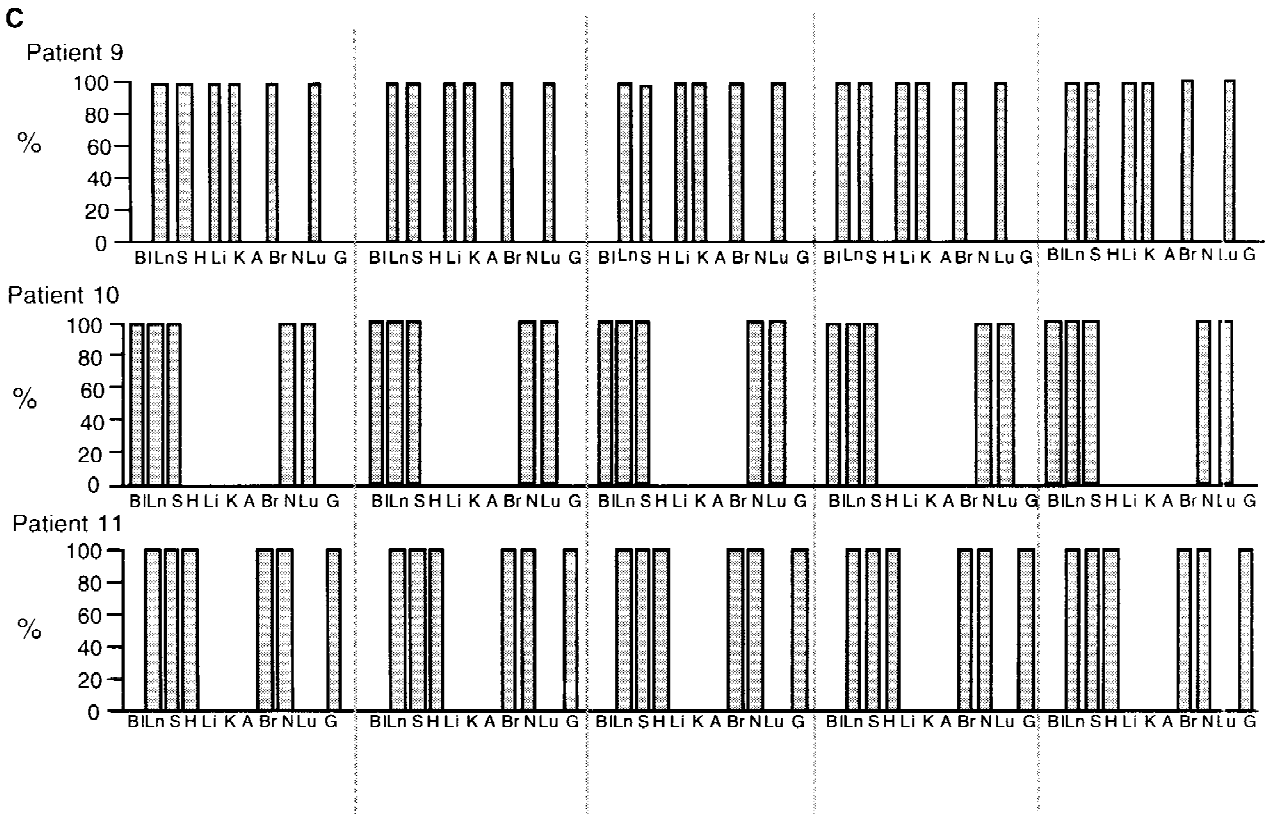


Fig. 3 Continued.

evolve even when viral load was at the lowest level detected by our assay (less than 20 genomes/μg DNA).

DISCUSSION

We used a combination of molecular genetic methods to analyse the quantitative prevalence of ZDV resistance in multiple organs of patients dying with AIDS. Notwithstanding this, the results clearly demonstrate that the evolution of ZDV resistance is not uniform between different organs and that high level resistance in one organ system such as the lymphoreticular system is not always predictive of high level resistance in other organs. Relatively few studies have addressed this aspect of resistance. Recently, the detailed study of SI and NSI strains has revealed differences in the phenotypes of strains present in multiple organ at post mortem (McGavin et al., 1996) and the genotypic analysis of a relatively small numbers of patients has shown disparities in the RT quasispecies present in brain and other organs (Ball et al., 1994; Wong et al., 1997). Our findings are therefore consistent with and extend substantially these previous published studies. In addition, we have complemented the genotypic resistance studies by assessing viral load differences between organ and its relationship with the presence of resistance. For individual patients viral load was consistently higher in lymph node and spleen compared to other organs. These data are consistent with the data reported by Donaldson et al. (1994) and emphasise the central role of the lymphoid tissues to HIV pathogenesis (Pantaleo et al., 1993; Nuovo et al., 1994). In organs not usually associated with HIV pathogenesis eg: liver, adrenal, elevated HIV-1 proviral loads were also observed in some individuals. Inspection of these organs by histology frequently revealed monocytic or lymphocyctic infiltrates which presumably accounted



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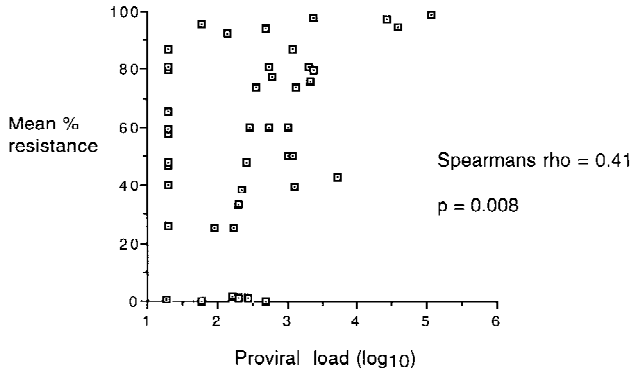

Fig. 4. Scatter diagram relating the $\log_{10}$ proviral load to the mean percentage of resistance codons present in the same sample for all patients exposed to ZDV. The mean percentage of resistance codons were calculated by summing the fraction of mutant sequences at each codon analysed and dividing by the number of codons included in the analysis. The latter controlled for instances in which the point mutation assay did not yield a result for one or more codons. The six samples with very low levels of mean resistance were all collected from patient 9 who received ZDV therapy only intermittently.

for this elevated virus. However, the veracicity of this facile explanation is questionable since other organs where such infiltrates were observed were frequently negative for HIV-1 proviral DNA. There was a weak relationship between high viral load and concurrent high level resistance. Whether this relationship reflects the growth advantage of the resistant genotypes in the presence of ZDV resulting in a higher tissue burden or higher viral loads at the initiation of therapy being more likely to generate resistance cannot be answered in this study.

In the majority of organs in which proviral load was measured, the distribution of wildtype/mutant alleles at ZDV resistance codons was also determined. However, in some instances the point mutation assay failed to produce results eg: codons 215 and 219 in the kidney of patient 5. DNA sequence analyses of these samples revealed the presence of multiple point mutations within the probe binding sequence which resulted in abrogation of probe binding (data not shown).

The underlying mechanisms which account for the different distributions of ZDV resistance mutations require consideration. Three explanations, which are not necessarily mutually exclusive, can be offered. Firstly, high level HIV-1 replication in organs generates sufficient diversity to allow the production of mutants which possess a selective growth advantage in the presence of ZDV (Nuovo et al., 1994). In contrast, organs in which viral replication is lower generate diversity and hence resistance genotypes less quickly. This explanation is consistent with the statistical correlation between viral load and resistance but does not account for the prevalence of resistant genotypes in samples with low viral loads. Second, the infection of non-lymphoid tissues has occurred at an early stage of HIV disease (with strains sensitive to ZDV) and these latent genomes persist at a low level of replication. Re-

activation of these strains in the target organ does not then occur until the later stages of infection leading to a phylogenetic disparity between strains resident in different organs irrespective of viral load (Coffin et al., 1995). Thirdly, ZDV penetration into multiple organs and/or its phosphorylation differs so that lower intracellular levels of ZDV triphosphate result in a reduced selective advantage for strains carrying the resistant genotype. At present, it is difficult to determine which of these possibilities predominates, although data are available indicating that ZDV anabolism can differ between organs (Pemo et al., 1992; Howe et al., 1992) and for the compartmentalised evolution of HIV strains (Ait-Khaled et al., 1995; Howe et al., 1992; Ait-Khaled et al., 1994; Pang et al., 1991; Ball et al., 1994; Wong et al., 1997) especially in the CNS (Ait-Khaled et al., 1995; Power et al., 1994; Cheng-Meyer et al., 1989).

To our knowledge, the analysis of ZDV resistance in the context of viral load has not been undertaken in multiple organs of patients exposed to any currently used anti-retroviral therapy. Indeed, such analysis may be difficult to perform at the present time due to the complications of overlapping resistant profiles in patients receiving multiple drug combinations. These data therefore have implications for the therapy of HIV-1 infection with newer anti-retroviral agents. For example, the presence of resistance in the blood may mislead clinical investigators into believing that HIV infection in other organs e.g. the brain is not being inhibited. This has important consequences since long term ZDV has been shown to reduce neurocognitive defects in HIV-1 infection (Baldeweg et al., 1995), and suggests that controlled clinical trials of alternative drugs versus continuing initial therapy are required together with appropriate clinical assessment. Meanwhile, further work is required to determine when resistant strains disseminate to internal organs and the consequences of disparate resistance profiles on disease progression.

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